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STUDIES ON ACQUIRED TYPHOID IMMUNITY *

F. W. HACHTEL AND H. W. STONER

From the Bacteriological Laboratory of the State and City Boards of Health, Baltimore

The experiments reported in this paper were undertaken primarily to determine whether or not immunity following infection or inoculation is due to a training or an exercise of the cells in the production of antibodies so that a later invasion will cause a more rapid or a more prolific formation of immune bodies, with a consequent destruction of the invading organism before it can gain a foothold.

This work, which we started 6 years ago, is not yet completed, but it is far enough advanced to warrant a statement of the results obtained. Our studies thus far have been limited to the effects of inoculation with typhoid vaccine and later re-inoculation with either dead or living typhoid bacilli. The entire subject falls into 2 parts: (1) An investigation of the changes produced in the blood serum by immunization, as determined by a study of the amounts of agglutinin, bactericidin, and opsonin in the blood serum of rabbits before, and at regular intervals after, inoculation. (2) An investigation of the effects of re-inoculation as determined by (a) a comparison of the amounts of agglutinin, bactericidin, and opsonin present in the blood serum of rabbits before, and at regular intervals after, the injection of antityphoid vaccine, with the amounts produced after re-inoculation of the animals with similar doses of the identical vaccine after the immune bodies have disappeared from the blood; and, (b) a comparison of the amounts of agglutinin, bactericidin, and opsonin found in the blood serum of rabbits receiving sublethal doses of virulent typhoid bacilli with the amounts found in the blood of previously immunized rabbits after they have received similar sublethal doses.

HISTORICAL REVIEW

Wollstein¹ studied the duration of the immune bodies present in the blood after typhoid inoculation, and found that in a series of 24 inoculated persons the bactericidin reached its greatest height 1 month after the 3rd inoculation, to fall rapidly during the next 2 months. Of 19 cases studied longer, 8 were negative for bactericidin 10 months after inoculation, and 15 after 13 months. The agglutinin disappeared even more quickly, for after 4 months only 2

* Received for publication March 3, 1916.

¹ Jour. Exper. Med., 1912, 16, p. 315.

agglutinated the typhoid bacillus in a dilution of 1:160, and no agglutinin was present 13 months after vaccination.

Cole² found that a guinea-pig which had been injected with typhoid bacilli formed antibodies more rapidly on being re-injected, while a control animal responded slowly, with a less abundant formation of antibodies.

Moon³ studied the agglutinin in human subjects who had been previously inoculated with antityphoid vaccine or who had suffered previously from an attack of typhoid fever. To quote from his article: "As shown by these curves there is a distinct difference in the rapidity with which human beings who have previously been immunized form antibodies as compared with those who have not. It would seem as if the previously immunized person remains more sensitive to the antigenic influence of typhoid bacilli and responds more quickly by the production of antibodies when the bacilli are introduced into the system. Such a result is in keeping with our knowledge of the phenomena of allergy. That this condition would contribute to the resistance against typhoid and the readiness with which the body would overcome the bacilli is easily understood."

TECHNIC

Agglutination.—The agglutination tests were made by the microscopic method. A 24-hour peptone-solution culture of a very sensitive strain of a typhoid bacillus that had been in the laboratory for several years was used. This organism was one of those present in the polyvalent vaccine employed in all the experiments. The serum, unless otherwise stated, had been heated beforehand for one-half hour at 56 C. Only those dilutions were considered positive which caused complete clumping with cessation of all motility within 2 hours. All tests were controlled by a hanging drop of the peptone culture.

Bactericidin.—The bactericidin was determined in vitro by a slight modification of the method first described by M. Neisser.⁴ In each of a series of small test tubes was placed 0.25 c.c. of a dilution (a different dilution in each case) of inactivated serum from the animal that was to receive, or had received, the inoculations of antigen. To each tube was added 0.5 c.c. of a 1:10,000 dilution of a 24-hour broth culture of the typhoid bacillus that was employed in the agglutination tests. The dilution of the serum and that of the typhoid culture were always made by the addition of 0.85% sterile salt solution. A dilution of complement was prepared by mixing 1 c.c. of fresh rabbit serum, 6.2 c.c. of salt solution, and 4.8 c.c. of sterile broth. To each tube 0.25 c.c. of this diluted complement was added, making the total quantity 1 c.c. The tubes were thoroughly shaken and incubated at 37 C. for 3 hours. They were again thoroughly shaken and the contents of each tube poured into a Petri dish and mixed with 10 c.c. of melted neutral agar that had been cooled to 40 C.

For each series of tubes 3 controls were made. First, a typhoid control, containing 0.5 c.c. of the diluted typhoid culture, 0.1 c.c. of sterile broth, and 0.4 c.c. of salt solution. This immediately after being made was poured into a Petri dish and mixed with neutral agar. It represented the number of typhoid bacilli planted in each tube. Second, a 3-hour control was made in the same manner as the typhoid control, but incubated with the tubes containing the serum to show the increase in the number of bacteria that occurred during the 3 hours. The 0.1 c.c. of broth added to each of these controls

² Ztschr. f. Hyg. u. Infektionskrankh., 1904, 66, p. 367.

³ Jour. Infect. Dis., 1914, 14, p. 56.

⁴ Ehrlich: Studies on Immunity, 1906, p. 348.

equalled the amount in the diluted complement of the other tubes. Neisser lays stress on the presence of the broth in these experiments, claiming that "it suffices to balance disturbing variations of the osmotic pressure." Third, a complement control was set up, consisting of 0.25 c.c. of diluted complement, 0.5 c.c. of the diluted typhoid culture, and 0.25 c.c. of salt solution. This was incubated with the other tubes and usually showed fewer colonies than the 3-hour control. The difference in the number of colonies represented the inhibitory action of the complement-bearing serum.

All plates were incubated for 48 hours, at the end of which the colonies on each were counted. Where the number was large we counted them with the low-power lens; in such cases the number of colonies in each of several different fields was determined, these numbers averaged, and the result multiplied by the number of fields on the plate.

This bactericidal test, as pointed out by Neisser, is subject to wide variations, and under no circumstances can it be considered very sensitive or accurate. A decrease in the number of colonies may be due to agglutination of the organisms. It is therefore necessary to make several dilutions of the amboceptor, and to regard as bactericidal only those that cause a marked diminution of colonies while higher or lower dilutions show large numbers of bacteria. The more concentrated solutions of amboceptor often fail to produce any bactericidal effect owing to an excess of the intermediary body and a consequent deflection of complement (Neisser-Wechberg phenomenon).

While Neisser estimated the bactericidal effect by dividing his plates into those containing no colonies, those containing 100, and those containing 1,000 and innumerable colonies, we have adopted the following arbitrary standard of what we believe may be considered bactericidal action: Since, other things being equal, the difference between the number of colonies on the 3-hour control and that on the complement control was due to the inhibiting effect of the normal rabbit serum, we determined in each instance the percentage of this reduction, deducted a proportionate number from the "typhoid control," and then regarded as bactericidal only those dilutions that gave plates containing not more than one-fiftieth the number of colonies on the corrected typhoid control. For example, if the 3-hour control plate contained 100,000 colonies, the complement control plate 75,000, and the typhoid control plate 20,000, then the normal serum inhibited 25,000, or 25% of the total number of bacteria in the 3-hour control. As the typhoid control contained 20,000 bacteria, each tube in the series was seeded with this number of organisms; but if 25% were restrained by the action of the complement-bearing serum there remained only 15,000 organisms to be acted on by each dilution of antityphoid amboceptor. Hence only the tubes that contained 300 colonies or less would be considered to show the effects of typhoid bactericidin.

We believe that this gives a generous margin for all the factors that might influence results. Altho agglutination may cause some error, the charts demonstrate that many of the sera were bactericidal at much higher dilutions than those at which they were agglutinative. The dilutions of bactericidin given in the tables are those of the amboceptor before it was placed in the tubes; as each of these contained 0.25 c.c. of this serum together with 0.5 c.c. of the culture and 0.25 c.c. of complement, the actual dilution of each immune serum was 4 times greater than that given in the charts.

Opsonin.—In our opsonic estimations we again used an arbitrary standard. The dilution method, modified after Neufeld, was employed as follows: An emulsion of the same strain of the typhoid bacillus as that used in the

agglutination and bactericidal tests was made by washing off with normal salt solution the 24-hour growth on an agar slant. Equal quantities of this emulsion, of leukocytic cream, and of one of the various dilutions of serum were mixed together in capillary tubes, which, after sealing, were incubated for 20 minutes. The ends were then broken and smears made and stained with Jenner's stain. The dilutions that caused 50% or more of the leukocytes to be phagocytic were regarded as being opsonic. The dilution as expressed in the text and the charts is that of the serum before the addition of bacterial emulsion and leukocytic cream; the actual dilution is therefore 3 times as great. Our experience with the opsonic tests was far from satisfactory; phagocytosis occurred only at dilutions much lower than those causing agglutination and this naturally would lead to erroneous results as the fields became obscured by the agglutinated bacilli.

The charts show the highest dilution at which each of the immune substances was active on the various days on which the blood was collected. The figures in the left-hand margin of each of these charts correspond to a position half-way between the lines.

I

In the study of the amount of agglutinin, bactericidin, and opsonin in the blood serum of rabbits before, and at regular intervals after, the inoculation with antityphoid vaccine, 11 rabbits were inoculated with 3 or 4 doses of typhoid vaccine. The size of the dose and the intervals between injections varied, but in most instances from 7 to 10 days elapsed between the successive inoculations.

A polyvalent vaccine made with 10 different strains of *B. typhosus* was used in all the experiments. The injections were made subcutaneously in the region of the groin after the hair had been shaved off, and the area washed with a solution of mercuric chlorid followed by sterile water.

Before collection of the blood, the ear was shaved and scrubbed with soap and water, sterilized with bichlorid of mercury and sterile water, and dried with sterile gauze. One of the more prominent blood vessels of the ear was punctured by a small blood stylet and the blood collected by means of a Wright capillary pipet. Bleeding was facilitated by placing under the abdomen of the rabbit a rubber bag filled with warm water. As soon as collected the blood was centrifugated and the serum inactivated by heating at 55 C. for one-half hour. If the blood was not used immediately, it was kept at a temperature of 10 C. until needed.

The 3 following experiments illustrate this section of the work:

Rabbit B.—This rabbit was first inoculated with 100,000,000 dead typhoid bacilli, 8 days later with 200,000,000, and again after 8 days with 300,000,000 dead bacteria. Blood was collected before inoculation and daily afterward; also on the 1st, 3rd, 5th, and 7th days after the second and third inoculations; 2 weeks and 1 month after the last inoculation, and at monthly intervals thereafter. The serum before inoculation agglutinated at a dilution of 1:4, but was not bactericidal. The curve for the antibodies following inoculation is shown in Chart 1.

Rabbit D.—This rabbit was inoculated with doses of 200,000,000, 400,000,000 and 600,000,000 dead typhoid bacilli, respectively, at intervals of 1 week. Blood

was collected before the first inoculation and every day for a week succeeding; on the 1st, 3rd, 5th, and 7th days after the second; and on the 2nd, 3rd, and 6th days after the third; also at the end of 2 weeks and after the 1st, 2nd, 3rd, 4th, 5th, 8th, and 9th months following the last injection. Before immunization the serum agglutinated at a dilution of 1:10, was not bactericidal, but was opsonic when undiluted. The result of the inoculations is shown in Chart 2.

Rabbit H.—The animal was injected at weekly intervals with doses of 250,000,000, 500,000,000, and 750,000,000 dead typhoid bacilli, respectively. Blood was collected before the first inoculation and daily following; on the 1st, 3rd, 5th, and 7th days after both the second and third inoculations; and also 2 weeks after the last, and then at monthly intervals. Before immunization the serum agglutinated at a dilution of 1:4 and was both bactericidal and opsonic when undiluted. Chart 3 shows the result of the experiment.

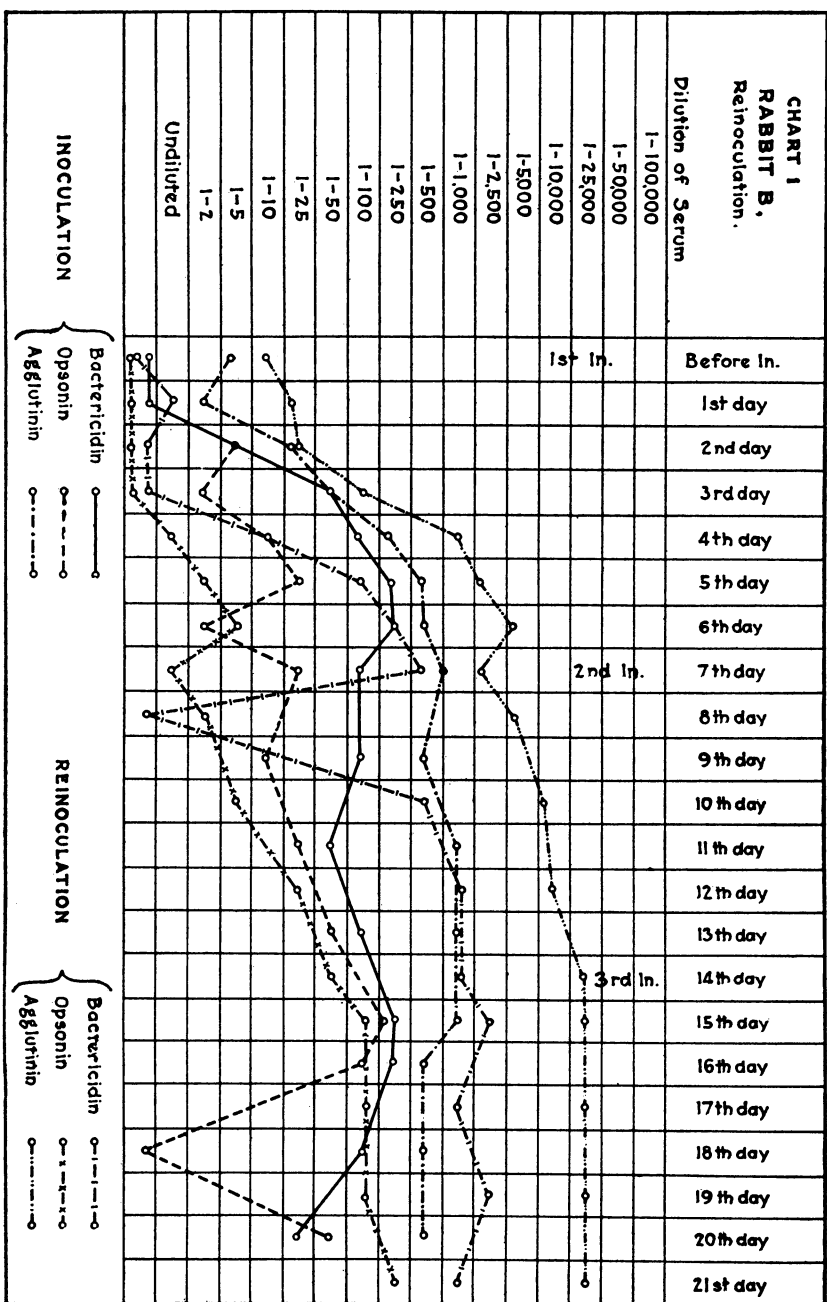
The 11 experiments with rabbits showed that the antibodies produced after the injection of antityphoid vaccine disappeared from the blood in from 6 to 340 days; on the other hand, figures indicate that the immunity conferred by inoculation of human beings usually lasts several years.

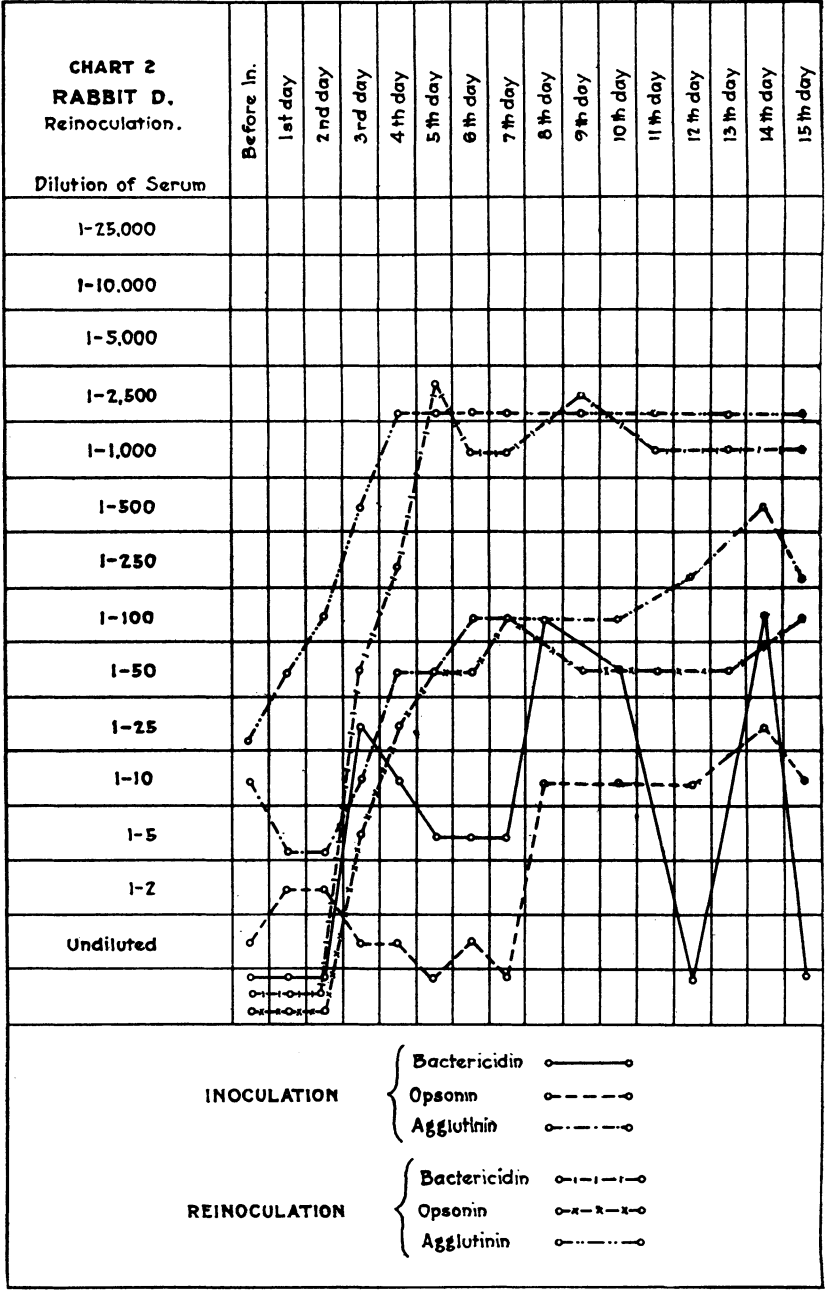
II

The following experiments were made to determine whether or not the injection of dead typhoid bacilli causes a sensitization of the cells concerned in the production of the immune bodies. As before stated, this problem was approached by a comparison of the amounts of agglutinin, bactericidin, and opsonin in rabbit serum before, and at regular intervals after, inoculation with antityphoid vaccine, with the amounts formed after re-inoculation with similar doses of the same vaccine after the antibodies had disappeared from the blood. Six rabbits were re-inoculated with similar doses of the same vaccine with which they had originally been immunized. The comparison between the antibody-formation after inoculation and that after re-inoculation is shown in Charts 1 and 3.

A second approach to the subject was made in a comparison of the amounts of agglutinin, bactericidin, and opsonin appearing in serum of rabbits receiving sublethal doses of virulent typhoid bacilli with the amounts appearing in the blood of previously immunized rabbits receiving similar sublethal doses.

Three rabbits were inoculated with a sublethal dose of a virulent culture of *B. typhosus* and one previously vaccinated rabbit was inoculated with an equal amount of the same typhoid culture. Rabbit D was selected to control the inoculations of sublethal doses of living typhoid cultures because, as measured by antibody-production, it had





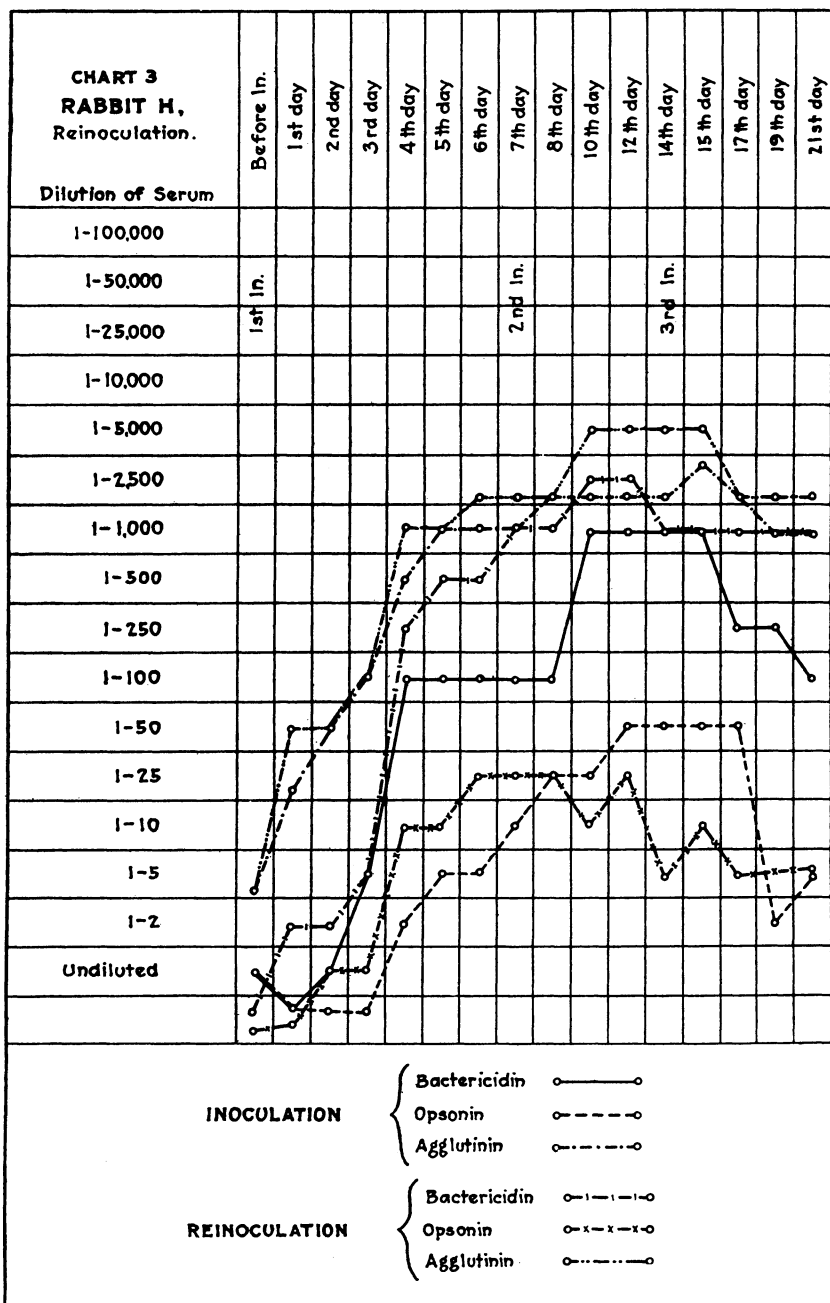


TABLE 1
TIME ELAPSING BEFORE THE MAXIMAL FACTOR WAS ATTAINED BY EACH OF THE ANTIBODIES
FOLLOWING THE PRIMARY AND SECONDARY SERIES OF INOCULATIONS

Rabbit	Days After Inoculation			Days After Re-inoculation		
	Agglutinin	Bactericidin	Opsonin	Agglutinin	Bactericidin	Opsonin
A	27	27	52*	16	26	22
B	11	5	15	14	15	21
C	16	7	10	18	16	
D†	14	8	14	4	5	7
F	21	21	21			
G	11	8	15			
H	15	10	12	10	10	6
J	15	21	7			
K	27	27	7			
L	8	6	6	6	8	19
M	14	14	6	6	7	7

* No opsonic determinations were made from the 13th to the 52nd day.

† This animal was re-inoculated with sublethal doses of living typhoid bacilli.

TABLE 2
TITER OF MAXIMAL FACTOR FOR EACH OF THE ANTIBODIES AFTER THE PRIMARY AND SECONDARY
SERIES OF INOCULATIONS

Rabbit	First Series of Inoculations			Second Series of Inoculations		
	Agglutinin	Bactericidin*	Opsonin*	Agglutinin	Bactericidin*	Opsonin*
A	1:1000	1:1000	1:25	1:2000	1:1000	1:100
B	1:1000	1:250	1:100	1:25000	1:2500	1:250
C	1:3000	1:250	1:50	1:10000	1:1000	
D†	1:500	1:100	1:25	1:2000	1:2500	1:100
F	1:3000	1:50000	1:500			
G	1:3000	1:10000	1:1000			
H	1:3000	1:1000	1:50	1:5000	1:2500	1:25
J	1:3000	1:5000	1:25			
K	1:4000	1:2500	1:100			
L	1:2000	1:500	1:10	1:1000	1:2500	1:50
M	1:50000	1:50000	1:50	1:100000	1:100000	1:2500

* The dilution of the bactericidin and of the opsonin is expressed in terms of that of the serum added to the other substances: the final dilution in each case would be 4 times and 3 times these figures, respectively.

† This animal was re-inoculated with sublethal doses of living typhoid bacilli.

TABLE 3
TIME ELAPSING BEFORE MAXIMAL FACTORS WERE ATTAINED FOLLOWING INOCULATION AND TIME
ELAPSING BEFORE THE SERUM REACHED THE SAME ACTIVITY, AS GAUGED
BY DILUTION, AFTER RE-INOCULATION

Rabbit	Days After Inoculation			Days After Re-inoculation		
	Agglutinin	Bactericidin	Opsonin	Agglutinin	Bactericidin	Opsonin
A	27	27	52*	5	26	18
B	11	5	15	4	6	Between 10 and 21
C	16	7	10	Between 10 and 12	12	
H	15	10	12	Between 8 and 10	7	†
L	8	6	6	†	5	5
M	14	14	6	Between 5 and 6	5	Between 3 and 4

* No opsonic determinations were made from the 15th to the 52nd day.

† The opsonin after re-inoculation did not, during the course of the experiment, reach the same maximal factor.

† The agglutinin after re-inoculation did not, during the course of the experiment, reach the same maximal factor.

developed a very high grade of immunity following the primary inoculations, and it had been immunized longer than any of the other animals.

The following experiment shows the result of the injection of a sublethal dose into an unvaccinated rabbit, while Chart 2 clearly shows the result for the vaccinated animal.

Rabbit W.—Inoculated subcutaneously with 5 c.c. of a 24-hour broth culture of living typhoid bacilli. Blood was collected before the inoculation, and on the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 9th, 11th, 13th, and 15th days thereafter. The serum did not cause agglutination before inoculation nor until the 4th day following, when clumping occurred at a dilution of 1:20. From the 6th until the 9th day the agglutinin increased steadily, being active at a dilution of 1:1,000 on the 9th day; from this time until the termination of the experiment there was no further rise in the curve.

No bactericidin was demonstrable in the serum of Rabbit W before inoculation. Seven days later the bactericidin was active at a dilution of 1:2 and from this time it increased steadily until the 13th day, when it was active at a dilution of 1:250; no difference was noticed on the 15th day, the last day on which blood was drawn.

No opsonin was found in the serum until the 11th day after inoculation, when phagocytosis occurred at a dilution of 1:5; on the 13th and 15th days the opsonin was active at a dilution of 1:10.

SUMMARY AND CONCLUSION

Of the 15 rabbits used in this work, all save 1 showed the presence of normal agglutinin in their sera; 7 of the sera were agglutinative at a dilution of 1:4, 4 at 1:10, and 1 at 1:20. Few of the animals, however, had normal bactericidin or opsonin in their sera. In no instance was bactericidin demonstrable in a serum diluted higher than 1:5, while opsonin could be demonstrated only when undiluted serum was used.

The agglutinin was usually the first of the immune bodies to increase following inoculation and it generally persisted for a longer time than either of the other two. The bactericidin, as a rule, was the second to increase and was usually present for a longer time than the opsonin.

With 2 exceptions the agglutinin attained its highest activity, as gauged by dilution, several days earlier after re-inoculation than after inoculation, and, with 1 exception, it reached much earlier the degree of activity corresponding to the maximal dilution factor after inoculation. In illustration of these statements Tables 1 and 3 are worthy of attention.

While it is evident from an analysis of the results in the cases of Rabbits A, B, C, H, L, and M—the animals re-inoculated with typhoid vaccine—that the bactericidin reached its maximal factors on the average a few days earlier following the primary series of inoculations, it must be remembered that the maximal factors attained after the 2nd series were almost invariably greater, as is clearly to be seen in the tables.

Again, the average maximal factor for each of the immune bodies was greater following re-inoculation than following the primary injections; in the case of the agglutinin, 1:23000 as compared with 1:10000; of the bactericidin, 1:18000 as compared with 1:9000; and of the opsonin, 1:585 as compared with 1:65.

Different grades of immunity, as measured by the antibodies considered, were produced in the different animals irrespective of their size, of the dose of vaccine employed, and of the intervals between inoculations.

There was no definite relation between high maximal factors of the antibodies and the length of time after inoculation that these bodies could be demonstrated in the serum, since in some instances the immune substances disappeared much earlier from the serum of animals which reacted at high dilutions than they did from the serum of those which reacted at comparatively low dilutions.

The experiments were too few to form the basis of any dogmatic statement, but it would seem that the immunity following inoculation is due to a training of the cells in the production of antibodies so that afterward they yield these more prolifically.